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RECENT ADVANCEMENTS AND BIOLOGICAL MANAGEMENT OF *FUSARIUM UDUM*: A CAUSATIVE AGENT OF PIGEONPEA WILT

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ABSTRACT

Fusarium udum Butler is an important soil borne pathogenic fungi causing wilt disease in pigeonpea. The disease is predominant in all major pigeonpea growing areas throughout the world, and causes 30-100% yield loss, where resistance sources are not available. The seedling stage is more prone to wilt infection, but the visible symptoms mostly appear at different growth stages of host plants based on the severity of infection. Though a number of well accepted techniques are available for resistance screening of *F. udum* wilt in pigeonpea, but most of the resistance sources are prevalent disease at early stage of plants. The incidence and relative importance together of this pathogen with present understanding of its interactions with host plants are of great concern. With the contemporary and traditional management practices adopted to control this disease, the increasing importance of development of ultimate resistance in elite pigeonpea cultivars with the help of advanced biotechnological strategies are listed and critically discussed. The present study aimed to discuss and find out a permanent solution by utilizing the best biotechnological approaches for the development of economically viable and ecologically sustainable effective management of wilt disease of pigeonpea caused by *F. udum*.

KEYWORDS: Disease, Fusarium, Management, Pigeonpea, Resistance, Wilt

INTRODUCTION

The world population is ever increasing and projected to increase by 1 billion in 2050 (9.6 billion) from the current population of 7.2 billion, according to a recent United Nations report (UNPAN, 2010). With a long term planning on sustainable genetic improvement of rice, wheat, maize like major staple crops, simultaneously it is much more important to gain a breakthrough on flawless production of proteinaceous foods to make a balance for diminishing global hunger and malnutrition.

Protein, the major 'building block' among all nutrients is presently available only 33% of its normal requirements in developing countries and making a big challenge to various nutritional development programs initiated to fulfill the targeted protein demand. Legumes can be considered as a best alternative of easily available protein resource and offer a 'handful quantity' of food proteins in the developing world with less cultivation care and low inputs. Among different leguminous crops, pigeonpea or red gram (*Cajanus cajan* (L.) Millspaugh) occupies a central place at world wide rainfed agriculture (Saxena *et al.*, 2010). At global level pigeonpea occupied 6.22 M ha in 22 countries and mostly in Asia and Africa. But surprisingly, India alone covers more than 70% area (4.65 M ha) among all pigeonpea growing countries (FAOSTAT, 2013).

Pigeonpea belongs to family Leguminosae is a major source of protein to about 20% of world population and also an abundant source of vitamins. In India, pigeonpea is the second most important food legume crop after chickpea. It is a multipurpose crop, being grown not only for grain but also for fuel and fodder. It is grown under a wide range of cropping systems on the Deccan Plateau (DP) in India (Allen and Lenné, 1998). The major restraint in pigeonpea production is considered to be biotic stresses where Fusarium wilt (FW) is considered as the most devastating disease followed by Sterility mosaic disease (SMD) and Phytophthora blight respectively (Pande *et al.*, 2011). In similar way, wilt disease is painstaking as a challenging problem in tomato caused by *Fusarium oxysporum* f. sp. lycopersici and *Rhizoctonia solani* resuling damping off, cankers, root rots, fruit decay, and foliage disease with serious economic loss (Morid *et al.*, 2012). The FW disease is reported to cause 30-100% yield losses in susceptible pigeonpea genotypes. Fusarium species are the most miscellaneous and widely dispersed plant-pathogenic fungi with broad host range, infecting both monocotyledonous and dicotyledonous plants and emerging pathogens of immune-compromised humans and other mammals (Ma *et al.*, 2010). In view of the importance of establishment of a sustainable disease management strategy, there is an urgent need to find out the best suitable approach from different control methods available at traditional and advanced level to combat against this disease.

Geographical Distribution and Severity

At the beginning of nineteenth century (1906), this devastating fungus under the genus Fusarium was first reported in pigeonpea crop by E. J. Butler in India and thus its nomenclature is given as *Fusarium udum* Butler (Karimi *et al.*, 2012). Moreover this disease is found to be abundant in India, east Africa and Malawi where in general yield losses crosses the border of 50% and beside these countries Bangladesh, Indonesia, Grenada, Myanmar, Mauritius, Nevis, Nepal, Venezuela, Trinidad and Tobago are known for field losses by FU (Reddy *et al.*, 2012 and Marley and Hillocks, 1996). Recently, this pathogen was recorded for causing severe disease in Southern Zambezia province under South Africa (Gwata *et al.*, 2006). Before this report, the disease was previously noted in African country when Munaa, a pigeonpea variety was first released shown Fusarium infection in Kenya in 1983 (Owuoche and Silim, 2010). Though the disease distribution also visualized in Tanzania and Uganda but the present distribution and incidence is not clearly known (Karimi *et al.*, 2012). As per as disease incidence and variability is concerned in Indian scenario, this pathogen displayed maximum occurrence (13.66 %) in Vidharbha followed by Marathwada region where maximum severity recorded in Marathwada region (90%) in the state of Maharashtra (Shinde *et al.*, 2014). Moreover, the wilt caused by Fusarium was effectively reported in the pigeonpea growing regions of Bihar, Jharkhand, Orissa and West Bengal with a considerable range of cultural, morphological and pathogenic variability in most isolates collected from affected areas (Kumar and Upadhyay, 2014). Genetic diversity and pathogenic variability of this pathogen was also recorded from different geographical locations representing seven states of India. Thirty *Fusarium udum* isolates with variable numbers were collected from the following states: Andhra Pradesh, Uttar Pradesh, Jharkhand, West Bengal, Haryana, Rajasthan and Punjab (Mesapogu *et al.*, 2012).

Symptomatology

As a soil-borne pathogen, the fungus makes an initial attack the host vascular system by entering through wounded root tips causes a gradual chlorosis on leaves, branches followed by collapsing and wilting of the root system. The infection without symptoms occurs in early seedling stage but clearly visible later in crop developmental stages. The

initial visible symptoms count in different ways such as interveinal clearing and loss of turgidity in leaves. These make them further a little chlorosis with bright yellow appearance before wilting (Jain and Reddy, 1995 and Reddy *et al.*, 1990).

It is also reported that, the typical symptoms of wilt disease in pigeonpea are distinguishable from the symptoms caused by other species of *Fusarium* genus dominant in other crops. Wilt symptoms in pigeonpea usually appear when the crop is in flowering or podding stage, but sometimes may be seen at seedling stage also. The most characteristic symptoms are browning or blackening of the xylem vessels and a purple band extending upwards from the base of the main stem. This band is more easily seen in pigeonpea with green stems than in those with colored stems. Partial wilting of the plant is also an indication of Fusarium wilt. When young plants (1-2 months) die from wilt, they may not show the purple band symptom, but have obvious internal browning and blackening (Allen and Lenné, 1998).

Pathogenesis and Disease Development

A complex interaction between plant and its fungal pathogen is an outcome of expression of both, plant defense genes as well as fungal pathogenesis related genes. The result of such a relationship is projected as either resistance or disease development in the plant. There are multiple events involved that lead to successful plant defense during pathogen attack. Further, these defense mechanisms are governed by an array of genes, which are either singly or synergistically, involved in plant resistance traits. Many defense related genes have been cloned and characterized in an attempt to elucidate the mechanism of defense upon Fusarium attack in various plant species, including pigeonpea (Gurjar *et al.*, 2012).

There are some typical factors have been identified which directly or indirectly influence the FW infection and disease development. It was clearly observed that, under different temperature regimes chickpea cultivar moderately resistant to F. oxysporium f. sp. ciceris showing variable responses to FW infection where they display a healthy development in between the temperature of 21–24°C, but fallen to highly susceptible condition when temperature rises to 25–27°C (Landa *et al.*, 2006). The growing nature of Fusarium population in soil is solely depends ecological nature and nutrient availability of soil. It was further established that the population enhancement of FW with disease development is proportionally related to high traces of nitrogen fertilization in agricultural soils (Groenewald, 2006). In another finding, it was also confirmed that water retentive nature of the soil and slightly acidic or alkaline soils with sand content more than 50% directly influence the favorable conditions of FW (Hillocks *et al.*, 2000). More over a recent scientific study on genetics of this pathogen revealed that the transfer of two lineage-specific (LS) genomic regions of chromosomes between strains of *F. oxysporum* lead to the conversion of a non-pathogenic strain into a pathogen and it directly ensures the strong involvement of evolution of fungal pathogenicity in disease development (Allen and Lenné, 1998).

Genetics of Resistance Mechanisms in Host Plant

A complex interaction between fungal pathogen and its host plant is an upshot of expression of both, plant defense genes as well as fungal pathogenesis related genes. The result of such a relationship is anticipated as either resistance or susceptibility in other word disease development in the plant system. A number of multiple events involved for successful plant defense during pathogen attack and of course the defense mechanisms are truly empowered by a group of genes, which are either singly or coordinately, involved in plant resistance functions. Many defense related genes like glucanases, chitinases and proteases have been cloned and characterized to understand their role and mechanism of defense upon Fusarium attack in various plant species, including chickpea (Giri *et al.*, 1998). Similar notable work of transcriptional

analysis was performed to identify a set of genes of interest in tomato plants infected with *F. oxysprum* f. sp. *lycopersici* (Fol) and Tomato Mosaic Virus (ToMV) where a large overlap was found in differentially expressed genes throughout the two incompatible interactions. However, Gene Ontology enrichment analysis evidenced specific categories in both interactions. Response to ToMV seems more multifaceted, since more than 70 specific categories were enriched versus the 30 detected in Fol interaction (Andolfo *et al.*, 2014). A number of well characterized or little known genes earlier reported to be involved in legume crops defense against Fusarium infection but still standing as an enigma for their role in pigeonpea like important crop plants.

The in-depth knowledge on genetics of resistance mechanisms is crucial for genesis of effective strategy for efficient transfer and stable function of such resistant genes into disease susceptible cultivars. A number of resistance theory against FW have been established and among them single dominant gene (Owuoche and Silim, 2010, Pandey *et al.*, 1996 and Kotresh *et al.*, 2006), parallel control of two complementary genes and major genes (Parmita *et al.*, 2005), duplicate genes, 2 genes and multiple factors (Okiror, 2002), and a single recessive gene (Jain and Reddy, 1995). Apart from dominant, recessive and complementary gene action (Kotresh *et al.*, 2006) are mostly popular. An important hypothesis demonstrated by Odeny in 2001 that wilt responsive genes are differentially controlled are totally depends on the origin of the resistance material experimented in a fastidious cross and the genetic background of which it belongs to (Odeny, 2001). Second most important factor can be considered as inoculation methods used which could make a range of variation in resistance depending upon their type. Some notable studies can be considered are wilt boxes (Okiror, 2002) or field (Jain and Reddy, 1995) which can act as a hub for the influence of environmental and edaphic factors on the disease severity and the expression of the resistance.

The genetics of FW resistance is still in an ambiguous mean, and much more genes proposed to be involved defense pathway by a single dominant gene to two complementary genes and might be even involvement of multiple genetic factors (Parmita *et al.*, 2005). Advancement of modern tool in the field of genomics especially molecular markers have revolutionized breeding in different cereal crops leading to the release of several improved cultivars/varieties with enhanced resistance/tolerance to biotic or abiotic stresses (Varshney *et al.*, 2006). Besides, further advancements have been furnished towards identification and tagging and pyramiding of wilt resistance (WR) genes. Although some old review on individual pulse crop is available, systematic review for wilt resistance at a single platform in the major pulse crops is lacking (Karimi *et al.*, 2012). The recent advances in modern biotechnology allow us to make so many broad ways to find out the causal factors involving in pathogenic infection to plants and simultaneously tell us the solution for the diagnosis and management of the said disease. Identification and quantification of pathogens can help in diagnosis, phylogenetics and suggesting the right management practices and storage possibilities for processors and growers. In addition to symptom analysis, culturing and microscopy modern molecular-based techniques play a major role in quick, reliable and accurate identification of fungal pathogens (Kumar *et al.*, 2015 and Choudhary *et al.*, 2013).

Act of Pathogen Related (PR) Proteins in Resistance against Fusarium

Disease resistance in plants depends on the capability of host to identify pathogens after immediate infection followed by initiation single or multiple patterns of defense mechanisms that restrict infection. The base level immunity in plants is acquired by the recognition of specific conserved microorganism-associated molecular patterns (MAMPs) by host specific pattern-recognition receptors (PRRs) that make a barrier on hosts against non-specialized pathogens. During initiation of infection, plants are able to establish immune responses by via pathogen receptors mostly Nucleotide binding-

ARC/leucine-rich repeat proteins (NBS-LRR) and also Receptor Like Proteins (RLP) and Receptor-Like Kinase (RLK) known as Pathogenesis Related proteins (PR) .These types of proteins are able to recognize the abundance of pathogen effector molecules and to make active effector-triggered immunity (ETI) (Sutherland *et al.*, 2013).

In other hand, fungal PR genes are related to events especially attachment of fungal spore and entry, infection with disease development and colonization of the host and so on. But after the immediate recognition of pathogenic MAMPs, the host cells trigger some very specific pathogenicity genes encoding such proteins engaged in suppression or disruption of host defense mechanisms. Some of well-known cell wall degrading genes encode enzymes like endo-polygalacturonase (pg1), pectate lyase (pl1), xylanase, exo-polygalacturonase (pgx4) and plant defence detoxifying enzyme like tomatinase, have been identified in F. oxysporum f. sp. lycopersici (Fol). During pathogenesis by Fusarium, a number of signaling genes expressed and some of them are mitogen-activated protein kinase (fmk1), G protein α subunit (fga1) and G protein β subunit (fgb1) (Andolfo *et al.*, 2014).

Among different PR protein genes, chitinase and β-1,3-glucanase considered as the most promising and strong defense related genes against many fungal pathogens. In response to fungal infection in many plants these enzymes are significantly induced and also activate induced resistance phenomena in hosts. It was also noticed that resistance is only possible for some fungal pathogens when combinations of these two enzymes are simultaneously worked rather than individual activity and in co-ordination with other defense related genes parallel increase in activities of the two enzymes is greatly increased (Saikia *et al.*, 2005).

Management and Control of Fusarium Wilt Disease

Resistance against a fungal pathogen can be achieved by single, race-specific resistance genes (R genes) effective for complete resistance, or by a group of minor genes coordinately working as a broad-spectrum incomplete resistance. Apart from biological and chemical control measures, without an in depth understanding of plant-pathogen interaction at genetic, histological and molecular level, complete management of fungal disease by bioprospecting of genes identified for conferring resistance is very much challenging. A number of control and management practices are come into the light of agricultural technology and most of them have pros and cons effect after implications. Therefore it is necessary to think about an innovative and potential approach by exploring the existing technologies to keep away the Fusarium infections resulting huge damage in legumes in sustainable way. Here is some mostly implemented management practices are elaborated with special care given about their potential application to minimize the post implementation side effects.

Cultural Control Method

A variety of cultural practices are applied in regular basis for creating a barrier against the Fusarium wilt infection in pigeonpea. Crop rotation could be useful as the best control measure with tobacco (*Nicotiana tabacum* L.), sorghum (*Sorghum bicolour* (L.) Moench) or castor (*Ricinus communis* L.) for at least three years is effective to eradicate the pathogen completely from the field. Another control measure could be cultivation of main crop in combination of one-year break with either sorghum or keep it as fallow and that can be reduce the chances of wilt infestation below 20%. Implement of nitrogenous in combination with farmyard manure or with *Crotalaria juncea* as green manuring also diminish the incidence of wilt up to a considerable range (Ingole *et al.*, 2005). Field treated with Solarization during summer season could be effective to reduce the population of Fusarium inoculums (Reddy *et al.*, 2012). However, there are

lacks of studies to understand the effect of cultural management practices with a future view of developing integrated disease management systems.

Chemical Control Method

Still it is the most common practices in agricultural commodities after the traditional cultural practices but the use of advanced chemicals day by day have reached to the level of best recommendation for the management of Fusarium wilt (Kotresh *et al.*, 2006). Seed treatment method with some chemicals such as equivalent mixture of benomyl and thiram (Reddy *et al.*, 2012), a combination of carbendazim + thiophanate (0.15 + 0.10%) (Mandhare and Suryawanshi, 2005) and *Trichoderma viride* formulation (4g) + Thiram (3 g /kg seed) (Verma and Rai, 2008) or another combination of *T. viride* (2 kg) formulation with 125 kg farm yard manure ha-1 (Perchepied and Pitrat, 2004) were found to be very effective in dropping the Fusarium wilt population. It was also reported that application of boron (Bo), zinc (Zn) or manganese (Mn) and methyl bromide (CH3Br) to the soil drastically reduce the disease event of Fusarium wilt (Maisuria *et al.*, 2008). Being a basic soil born pathogen, none of these fungicides are not only sufficient to give cent percent protection against Fusarium wilt disease but also cause broad spectrum side effects like killing of non-target beneficial soil microorganisms and environmental hazards (water and soil pollution) (Kotresh *et al.*, 2006).

Biological Control Method

Taking into consideration of the adverse and unpredictable effects of chemicals application in agricultural system implementation of biological agents acting as a part of same ecosystem and potential antagonist to most plant fungi is getting more importance in the advance stages of plant protection measures. Though a bunch of scientific reports came in front as functional biological measure but some of them are quite notable. These are mainly like showing of soils with fungal or bacterial antagonists (Karimi *et al.*, 2012 and Siddiqui, 2005), specifically rhizobacteria as biocontrol agents (Siddiqui and Shakeel, 2007 and Prasad *et al.*, 2002), *Trichoderma harzianum* in the form of oil amendment (22% -61.5% reduction of all fungal diseases) (Khan and Khan, 2002), root-dip inoculation with *Bacillus subtilis*, *Pseudomonas fluorescens*, *Aspergillus awamori*, *Aspergillus niger* and *Penicillium digitatum* (effective for management of *Fusarium oxysporum* in Tomato) (Anjaiah *et al.*, 2003), pigeonpea seed inoculation with *Pseudomonads aeruginosa* (Mahesh *et al.*, 2010). Beside these it is also suggested that integrated management (systemic fungicide, biocontrol agent and FYM) can be most powerful treatment for large scale management of FU (Pande *et al.*, 2012).

Screening of Germplasm and Lines of Pigeonpea for Resistance

This pathogen is so much stable that once it infests a field, the fungal spore may continue to exist in the soil micro environment for several years. Implication of cultivars/varieties or genotypes resistant to this pathogen is the most effectual measure for controlling the disease. The progressive threat of this disease in a cultivated pigeonpea variety can be diagnosed by the means of following studies (a) evaluation of reaction to Fusarium wilt and (b) under high disease pressure, agronomic performance of elite cultivars/lines/ germplasm or genotypes of pigeonpea to discover the best one for disease resistance (Gwata *et al.*, 2006).

The above study is currently getting popular because some land races/variety of pigeonpea has shown better response in resistance breeding programs against wilt of pigeonpea and there are no reports of immunity against this disease. Sources of resistance identified in one region do not perform with the same degree of resistance in other regions thereby indicating pathogenic variability in the fungus (Kumar and Upadhyay, 2014). Assessment of the effect of any

disease on yield of a crop is pre-requisite for preparing rational disease management program. Using fungicides and cultural practices for the management of the diseases under environmental conditions favorable to disease development is uneconomical and difficult to carry out. Of the available management approaches, host plant resistance (HPR) is the most reliable, economical and effective method for managing the diseases. Considerable efforts have been made by ICRISAT towards understanding the components of HPR such as biology and epidemiology, developing screening techniques, identifying resistance sources and utilizing these in breeding disease resistant lines (Park *et al.*, 2008). Use of resistant cultivars is the most practical and economical method for any disease management practices. However, in case of vascular wilt caused by *F. udum*, deployment of resistant varieties is not feasible due to the high genetic variability in the pathogenic population (Kumar and Upadhyay, 2014).

The genetics of FW resistance is still unclear, and numbers of genes hypothesized to be involved vary from a single dominant gene to two complementary genes and even involvement of multiple factors (Okiror, 2002). Several wilt resistant genotypes have been identified and reported where these sources of resistance can be used as resistant donors in pigeonpea wilt resistance breeding program. However, there is a need for better understanding of the inheritance of resistance, particularly in view of that fact that genotypes show different levels of resistance under field conditions. Some of the new sources of resistance reported against Fusarium wilt but there are still a lot of probabilities of getting improved and potential germplasm or indigenous genotypes at rural level by searching, collecting and evaluating such genotypes for better resistance by following standard inoculation method (Rispail *et al.*, 2013).

Advancement in Exploitation to Resistance: Biotechnological Aspects

- **Transcriptomics Approaches**

The preliminary step in defense response is the identification of the pathogenic molecule by specifically designed plant receptor protein followed by activation signal transduction cascades that consequently elicit the transcription of various plant defense genes (Barilli *et al.*, 2014). The unique advantage of gene expression studies is that it provides a local as well as global view about differentially expressed genes and metabolic pathways during plant-pathogen interactions and gives the best chance for identification of candidate resistant genes engaged in every steps of plant defense response (Ichinose *et al.*, 2001 and Matsui *et al.*, 2004). In the advanced area of molecular plant breeding, Marker assisted selection (MAS) can make a promising achievement by implying the knowledge of the defense responsive genes involved under fungal pathogen attack to legume plants and the possible altered expression of such candidate genes under transformation event can also be correlated with improved resistance . As very little or limited knowledge of gene sequences related to legumes and their pathogens are available in public domain, it made the molecular studies in legume-pathogen interactions quite difficult in recent past. Therefore, it is imperative to study in depth about the pattern of gene expression in response to a pathogen attack or elicitors in legumes by using such genes those have sufficient sequence information (Gao *et al.*, 2007, Rubiales *et al.*, 2015 and Wesley *et al.*, 2001). Alternatively the potential number of defense-related genes could be enhanced by generating cDNA (complementary DNA) libraries from plants under stress against pathogens inoculation or elicitor-treated tissues or cells. Another most potential technique is the application of macro or microarray designed by using orthologue sequences from other legumes in the format of unigenes, cDNAs, Expressed sequence Tags (ESTs) or resistance gene analogs (RGAs) in the query legumes like pigeonpea under specific fungal stress conditions. These technologies offer the discovery of such known or unknown transcripts that highly induced under pathogenic attacks and mostly linked to candidate resistant genes with an estimated level of expression.

As the microarray technique is limited with previous knowledge requirement of genome sequence, there are also some alternative approaches where a snapshot of differentially expressed transcripts can be achieved without previous genetic information and these are suppressive subtractive hybridization (SSH) and cDNA-amplified fragment length polymorphisms (AFLP) for identification of legume biotic stress responsive genes. Beside these the advancement of technology offers to explore the genomic sequence information with the help of newly adopted less expensive sequencing platforms (Ilumina (Solexa) sequencing, Roche 454 sequencing, Ion torren (Proton / PGMsequencing) and SOLiD sequencing) which not only facilitating de novo sequencing of transcriptome of any organism but also reduce the standard sequencing cost. In addition, their implementation in the area of legume biotechnology will disclose most of the genes that directly or indirectly related to defense responsive networks or pathways in individual legumes crops. In other way NGS technologies also reduce the complexity of transcriptome techniques including SSH, cDNA-AFLP, SuperSAGE (Serial analysis of gene expression) or MPSS (Massive parallel signature sequencing) in the way by increasing the identified transcripts amount without the need of cloning and Sanger sequencing. More recently RNAseq technique offers an additional taste to develop de novo transcriptomics that not allowing the sequencing of all transcripts expressed in a given situation but also it can generate the transition of transcript in term of expression of both plant host and the inoculated fungal pathogen in order to investigate plant-pathogen interactions study (Tadege *et al.*, 2008).

Though transcriptome profiling techniques across the genome can generate a huge number of differentially expressed genes population under pathogen attack but it is also challenging to distinguish such transcripts that are really associated with defense response and resistant phenotypes. It can be resolved by studying their co-localization with Quantitative trait loci (QTLs) and to carry out functional analysis though it required an excellent professional to perform and execute high-throughput reverse genetic approach in pigeonpea like legume crops. It could be feasible at biochemical and physiological level to conduct functional analysis of candidate genes and that can be performed by two ways of transformation based approaches, protein over-expression and promoter activity studies. In recent times, the accurate detection of functional activities of PR proteins and biotic stress induced genes is successfully achieved by different advanced molecular techniques like gene silencing technologies through RNA interference (RNAi) and virus induced gene silencing (VIGS) (Tadege *et al.*, 2009). Nevertheless, most of the legumes are difficult to adopt these approaches because of their success depends mainly on efficient genetic transformation protocols. As an alternative, a variety of mutation based functional genomics strategies have been developed, of which Targeting induced local lesion in genome (TILLING) and deletion-TILLING (de- TILLING) are mostly studied. These approaches basically deal with the discovery of mutants holding specific mutation in genes of interest from large mutant populations derived from fast neutron mutagenesis or saturating Tnt1-insertion mutagenesis and chemical mutagenesis (Hammond-Kosack and Jones, 1997 and Kanazin *et al.*, 1996). However this TILLING approach has not been applied to study disease resistance in legumes.

Resistance Gene Analogs (RGAs): A Promising Approach

Now a day's a number of resistance genes against different pathogens have been cloned from various plants of different genera and species. Surprisingly, majority of them share common domains in the putative proteins they encodes and of them leucine-rich repeat (LRR), nucleotide-binding site (NBS) and serine/threonine kinase domains are remarkable (Dangl and Jones, 2001). The existence of these common conserved regions in resistance genes across species provides an opportunity to isolate, clone and characterize novel resistance gene analogs in other plant species with the help of PCR based strategies (Caplan *et al.*, 2008). There are a numerous reports displayed the successful isolation and cloning of

functional disease resistance (R) genes encoding resistance to fungal, bacterial, viral, nematode and insect pathogens (Fondevilla *et al.*, 2008). The most common plant R genes are genes encoding nucleotide binding site-leucine rich repeat (NBS-LRR) and its immune receptors can identify specific pathogen-effector proteins and generate defense related signaling to stimulate defense response (McIntyre *et al.*, 2005). Thus the utility of RGA can ascertained by cloning of disease resistance (R) genes from diversified population of plant species followed by identification of amino-acid specific conserved domains among these genes and isolation of homologous sequences representing resistance gene candidate (RGCs or RGAs) by using conserved domains specific degenerate oligonucleotide primers designed (Chen *et al.*, 1998). Now RGAs can be used as representative fragments of R genes and now easily can be utilized for developing molecular markers for mapping of QTLs related to previously identified resistance loci (Huang *et al.*, 2004). Current advancement in molecular characterization of plant resistance genes (R-genes) has accelerated the development of very resistance gene analog polymorphism (RGAP) markers very specific pathogenic resistance gene. RGAP markers are mainly designed from the conserved domains including nucleotide-binding site (NBS), leucine-rich repeat (LRR) and protein kinase of resistance genes (Canovas *et al.*, 2004). Therefore, the RGA based molecular approach offers not only a commanding tool for the isolation of resistance genes candidate (RGCs) with simultaneous analysis of their structure and evolution but also contributed as a resource of useful marker source for MAS (Lee *et al.*, 2015).

Proteomics Approaches

The successful event of protein expression and its functional activity is solely depends on the degree of gene expression, post-transcriptional and post-translational regulations. Thus, there is a huge probability that all successful expression of mRNA derived transcripts does not always give successful protein accumulation and functions. As a result, expression profiling of gene transcripts is not only the way out to gets a clear picture of the mechanisms of plant-pathogen interaction and auto 'switch on and off' of resistance during pathogenic interference and it is equally important to correlate with the study of protein accumulation. The modern proteomic technologies offer a tremendous opportunity to establish large-scale protein profiling by means of quantitative and qualitative methods (Qin *et al.*, 2013). Conventionally, protein separation based on their mass and isoelectric points by electrophoresis and subsequent spectrometry techniques based protein identification like peptide mass fingerprinting or de novo sequencing are the mostly used comparative proteomics approach. In the other hand, chromatography-based peptide mixtures separation followed by their identification through mass spectrometry is totally independent from gel electrophoresis based separation techniques which is also getting popular (Nautrup-Pedersen *et al.*, 2010). More recently, shotgun proteomics deals with direct tandem mass spectrometric analysis which involves cell lysis based extended chromatographic separation and also another example of gel-free approach for protein identifications (Qin *et al.*, 2013). The above proteomics approaches have mostly being studied in legume crops especially for the establishment of sub cellular localization of target proteins and to develop the reference protein maps (Salavati *et al.*, 2012); seed protein content and subsequent modification during seed development (Palomares-Rius *et al.*, 2010); the study of symbiotic association of plants with rhizobium bacteria and mycorrhiza fungi (Zhang *et al.*, 2011). In contrast, the proteomic studies on changes happened due to pathogen attack in legumes are far lack behind other molecular advancements except one experiment concerned with the proteome response of chickpea - *Fusarium oxysporum* (Bourgeois *et al.*, 2011). More over the comparative proteomic approaches is highly potential to detect changes in various proteins under biotic stresses response though a few number of candidate resistance proteins are identified till date by using these proteomic studies. Therefore a lot of scopes are expected from these proteomic techniques that could allow to the discovery of such endogenous elements responsible for resistance to fungal diseases. In

addition the newly developed intra- or cis-genesis approaches offer an artificial and desirable modification of candidate proteins by means of genetic transformation techniques (Holme *et al.*, 2013). In future, the relevant applications of these existing approaches with continuous modification in comparative proteomics could make it possible to rising up a profound knowledge of legume resistance and regulation of protein function in response to pathogenic stresses (Wesley *et al.*, 2001).

CONCLUSIONS

A high variety of molecular methods have been used to detect, identify and quantify a long list of highly active plant defense responsive genes, enzymes proteins and some time transcription factors against pathogenic fungi. Additionally, these techniques only allow the detection and identification of important genes but how they are behaving at different expression levels during pathogen attack with variable symptoms under different developmental stages would not be well experienced by these molecular techniques. But current understanding of specio-temporal expression of these promising genes during pathogenesis by studying the different patterns of symptomatology in resistant vs. susceptible plant could be the vital factors for crop improvement with well developed resistance mechanism. In this way, recent biotechnological breakthrough is providing important information on transcriptome of plants to investigate stress responsive genes under different biotic stresses especially under fungal infection. Further genomic, proteomics and metabolomics studies are necessary to enlighten whole cellular processes related to biotic stress tolerance which can make a big lead for advance crop improvement.

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